FERTILIZATION OF FERTILIZED SEA URCHIN EGGS[1]

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Experiments by Tyler and Schultz (1932) on eggs of the echiuroid worm *Urechis caupo* showed that repeated fertilization was possible. In these experiments development of the eggs was blocked by treatment with slightly acidified sea water within three minutes after the first insemination. When returned to normal sea water at 15 or more minutes later these eggs failed to resume development although containing a spermatozoon. In fact they assumed the appearance of unfertilized eggs. Upon re-insemination a second sperm entered and development proceeded with typical polyspermic cleavage and the formation of abnormal embryos. These experiments have been confirmed on eggs of *Urechis unicinctus* by Kagawa (1952).

In the above-described experiments the possibility of re-fertilization was apparently confined to the first few minutes after fertilization and before the time at which membrane elevation occurs in this species (see Tyler, 1932). More recently it has been shown by Sugiyama (1951) that sea urchin eggs which have been mechanically deprived of their fertilization membrane can be re-fertilized (becoming heavily polyspermic) at considerably later stages provided they are exposed for a short time to Ca- and Mg-free sea water or to isotonic urea. The re-fertilization could be accomplished as late as the two-cell stage. The treatment with Ca- and Mg-free sea water or with isotonic urea usually was for five minutes at a short time after the initial insemination. Sugiyama also reported that if the eggs are put in Ca- and Mg-free sea water before the fertilization membrane rises the latter elevates as a thin structure. When returned to normal sea water two to five minutes later and sperm added the membranes sink to the surface of the egg and re-fertilization occurs.

Hagström and Hagström (1954a) confirmed the re-fertilizability of fertilized sea urchin eggs, using trypsin-treatment of the unfertilized egg to inhibit membrane elevation and Ca- and/or Mg-free sea water treatment of the fertilized eggs to render them susceptible to re-fertilization. In all these experiments the re-inseminated eggs tend to become heavily polyspermic. Evidently the eggs have not only become re-fertilizable as a result of the treatment but also they appear to have lost the ability to propagate the normal rapid block to polyspermy characteristic of the untreated unfertilized egg (see Rothschild, 1954).

Eggs that are activated artificially can also be entered by a number of sperm if the membrane is removed, as in the early experiments of Loeb (1913) and Moore

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(1916, 1917) or especially if treated with Ca- and Mg-free sea water as in the recent experiments of Ishida and Nakano (1947, 1950) and Nakano (1954).

We have confirmed and extended some of the above experiments. In addition we have found that it is possible to re-fertilize eggs of the sea urchin *Lytechinus pictus* and *L. variegatus* at later stages without any treatment other than removal of the fertilization membrane.

Materials and Methods

Eggs and sperm of the sea urchin *Lytechinus pictus* (Newport Bay, California) and *Lytechinus variegatus* (Alligator Harbor, Florida) were used in most of the experiments. Usually they were obtained by the method of KCl injection (Tyler, 1949). The sand dollar *Dendraster excentricus* was used in two sets of experiments, the eggs and sperm being obtained in the same way.

Mechanical demembranation was accomplished by means of a syringe and 20 gauge needle, the eggs being drawn through the needle at about two minutes after insemination when the fertilization membrane was almost fully elevated.

The trypsin employed in certain of the experiments was a crystalline preparation (Nutritional Biochemicals Corporation) and the papain was a non-crystalline preparation (Merck). The latter was prepared for use as described by Tyler and Spiegel (1956), 2 g. of the papain being extracted with 100 ml. 10^{-3} *M* Versene-sea water and filtered. One ml. of this is added to 100 ml. 0.2% L-cysteine hydrochloride (Merck) in 5×10^{-4} *M* Versene [5]-sea water and adjusted to pH 8.

The Ca- and Mg-free medium (see Tyler, 1953) consisted of a solution of 100 volumes of 0.55 *M* NaCl, 2.2 volumes of 0.55 *M* KCl, 10.3 volumes of 0.37 *M* Na_2SO_4 and ca. 0.6 volumes of 0.55 *M* $NaHCO_3$ to bring the solution to pH 8.

In all of the experiments, except where otherwise noted, the semen was initially diluted to 1% concentration or higher in 10^{-3} molar Versene in sea water and stored as such at the experimental temperature. The Versene was employed because of its ability to maintain the motility and fertilizing capacity of the sperm unchanged for prolonged periods (see Tyler, 1953). The experiments were all run at 17° C., except where otherwise noted.

Results

Re-fertilization of mechanically demembranated eggs after treatment with Ca- and Mg-free sea water

The experiments of Sugiyama (1951) with Ca- and Mg-free sea water treatment could be readily confirmed with eggs of *Lytechinus*. We found, too, that it was not necessary to re-inseminate immediately after return of the eggs to normal sea water. If the demembranated eggs are given a five-minute treatment with Ca- and Mg-free sea water shortly after the first fertilization and returned to normal sea water they retain their ability to be re-fertilized for at least 30 minutes. The results of two experiments of this type are given in Table I.

[5] Ethylenediamine tetraacetic acid. Acknowledgment is made to the Bersworth Chemical Corporation, Framingham, Mass. for a supply of this reagent.

TABLE I

Re-fertilization of eggs of Lytechinus pictus after mechanical demembranation and treatment with Ca- and Mg-free sea water for 5 minutes at 3 minutes after the first insemination

Time between first and second insemination	Percentage of polyspermy (0.1 ml. of 1% semen added per 5 ml. egg suspension)	
	Experiment 1	Experiment 2
Not re-inseminated	4	4
8 minutes	87	49
13 minutes	46	80
23 minutes	54	28
38 minutes	68	12

Re-fertilization of mechanically demembranated eggs without further treatment

As an additional control in the above-mentioned experiments the treatment with Ca- and Mg-free sea water was omitted and a high percentage of polyspermy was obtained upon re-insemination. A series of tests was therefore made of the re-fertilizability of eggs that had been subjected simply to demembranation and re-insemination. The results of five such experiments with eggs of *L. pictus* are listed in Table II. It is evident that fertilized and demembranated eggs can accept additional sperm as late as 40 minutes (at 17° C.) after the first insemination. The re-fertilizability is greater the shorter the time interval between the first and second inseminations, 100 per cent re-fertilization being readily obtained when the re-insemination is done within the first 10 minutes. Even at 15 minutes, as in experiment 5, the eggs can all be re-fertilized if sufficiently concentrated sperm is employed.

The data of the table also show that concentrations of sperm that give 100 per cent re-fertilization cause very little polyspermy when added as initial inseminates to the unfertilized eggs. It also appears from the data of Table II that the process of demembranation permits additional sperm of the initial inseminate to enter the eggs. This effect is, however, relatively slight as a comparison of the percentage polyspermy of the non-demembranated with that of the demembranated eggs in each of the experiments shows. Evidently most of the spermatozoa present in the initial inseminate are not capable of entering the demembranated eggs. Microscopic observation shows that a large proportion of the initially added sperm undergo the spontaneously reversing agglutination characteristic of the reaction with fertilizin in this species of sea urchins (*cf.* Tyler, 1948, 1955), and remain quite active thereafter.

Photographs of re-inseminated demembranated eggs are presented in Figure 1 along with those of non-demembranated and of demembranated and not re-inseminated, controls. In the sample re-inseminated at 10 minutes (Fig. 1e) all of the eggs are heavily polyspermic as shown by the clear areas representing the asters. The polyspermy here is too heavy to permit cleavage. In the various experiments, when the re-insemination is done early (10 to 16 minutes after the initial insemination) and with relatively large amounts of sperm (10^7 or more per ml. of egg suspension) practically all of the eggs show this high degree of polyspermy and failure of cleavage. From observations of the numbers of sperm asters it appears that five or more sperm can enter heavily re-inseminated eggs. In less heavily re-

TABLE II—*Re-fertilization of mechanically demembranated eggs of Lytechinus pictus at various times after fertilization*

Experiment No.	Time between first and second insemination (minutes)	Number of sperm ($\times 10^6$) per ml. of egg suspension		Percentage polyspermy determined at first cleavage (ca. 2 hours)
		First insemination	Second insemination	
1	Not demembranated	1.2	0	1
	Demembranated	1.2	0	3.5
	$2\frac{1}{2}$	1.2	1.2	53
	$4\frac{1}{2}$	1.2	1.2	51
	10	1.2	1.2	67
	20	1.2	1.2	20
	30	1.2	1.2	13
	40	1.2	1.2	35
2	Not demembranated	28	0	1
	Demembranated	28	0	5.6
	3	28	28	100
	5	28	28	100
	7	28	28	98
	10	28	28	96
	30	28	28	40
3	Not demembranated	2	0	2.5
	Demembranated	2	0	13
	5	2	6	100
	10	2	6	100
	20	2	6	81
	40	2	6	27
	Not dememb. −40	2	0	6
4	Not demembranated	1	0	3
	Demembranated	1	0	22
	10	1	0.4	37
	10	1	1	51
	10	1	4	100
	10	1	8	100
	40	1	0.4	32
	40	1	1	35
	40	1	4	76
	40	1	8	65
5	Not demembranated	49	0	2
	Demembranated	49	0	3
	8	49	49	100
	8	49	245	100
	15	49	49	68
	15	49	245	100
	30	49	49	11
	30	49	245	78
	40	49	49	17
	40	49	245	61

In experiments 1 to 4 the semen was diluted initially to 1% in $10^{-3}M$ Versene in sea water and stored for the later inseminations at that concentration. In experiment 5 the semen was diluted to 10% in ordinary sea water. Sperm counts were made in experiments 2, 4 and 5; sperm estimated in experiments 1 and 3 from dilution on the basis of a content of 2×10^{10} per ml. semen.

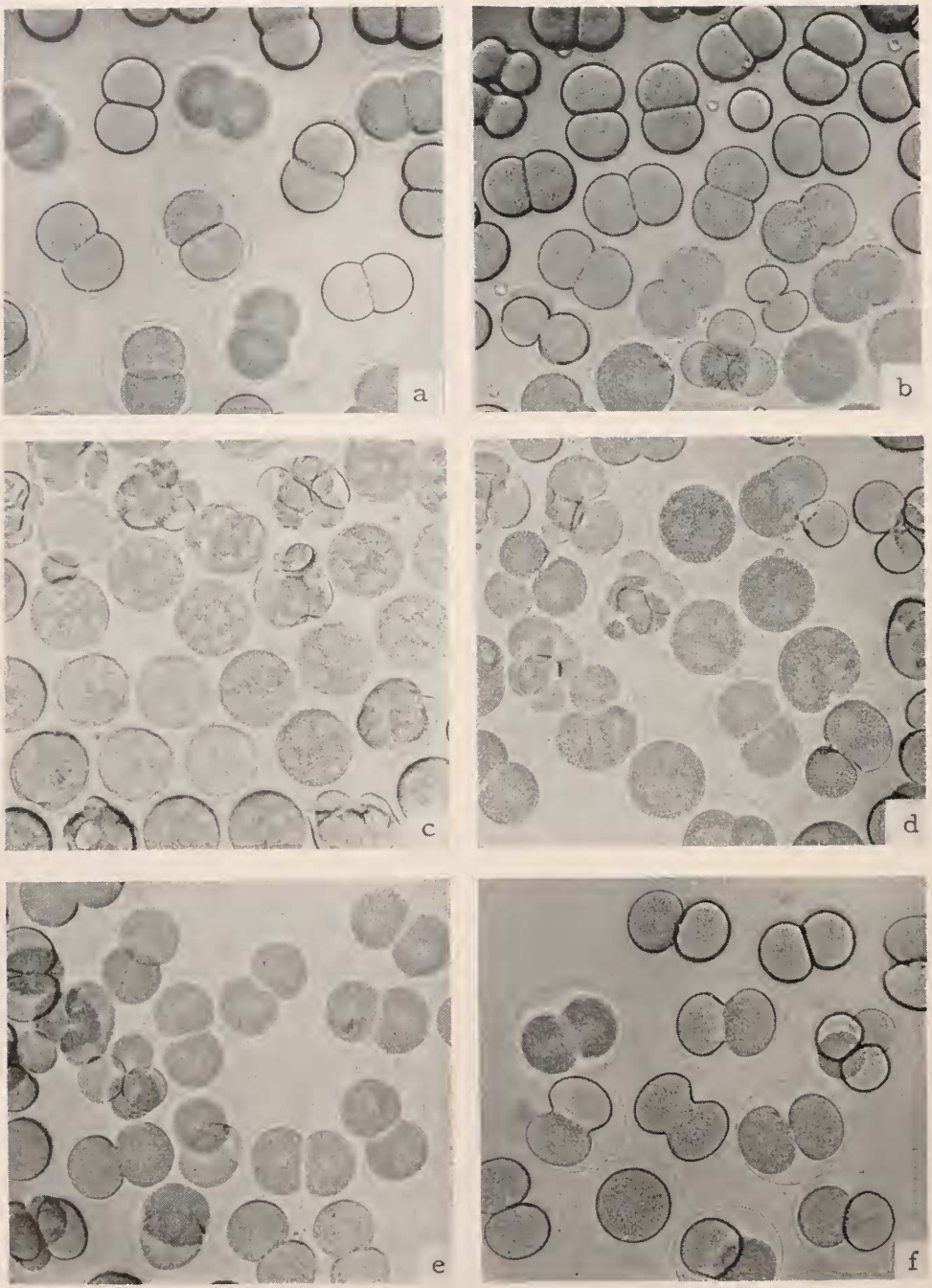

FIGURE 1. Re-fertilization of mechanically demembranated eggs of *Lytechinus pictus*. Insemination number 1 in 0.01%, number 2 in 0.03% semen. Eggs demembranated at one minute

inseminated, demembranated eggs, and in those re-inseminated at later times, the eggs that become re-fertilized generally accept fewer sperm and cleavage occurs in the form characteristic of dispermic or trispermic eggs (see Fig. 1d and e).

Two sets of experiments were performed with mechanically demembranated eggs of the sand dollar *Dendraster excentricus*. In one of these the re-inseminations at 10 and 30 minutes with 10^8 sperm per ml. gave 8.2 and 5.1 per cent polyspermy, respectively, as compared with 1.6 per cent in the non-reinseminated control. In the other experiment re-insemination with 2.2×10^7 sperm per ml. at 6 and 10 minutes gave 9.2 and 7.3 per cent polyspermy and 3.8 per cent in the controls. The results with lower concentrations of sperm and later times of re-insemination differed less significantly from the controls.

Experiments with mechanically demembranated eggs of *Lytechinus variegatus* gave results similar to those obtained with *L. pictus*. Two sets are presented in Table III which includes a comparison of eggs which had been subjected to Ca- and Mg-free sea water with eggs that were not so treated. In the first set the initial insemination was rather heavy (2% of semen which was originally diluted somewhat, perhaps ¼, by fluids shed with the semen). The non-demembranated, once-inseminated controls gave 10.5 per cent polyspermy. Demembranation alone at 1½ minutes resulted in an increase in polyspermy to 45 (Ca- and Mg-free sea water-treated eggs) and 58 per cent (untreated eggs), presumably due to re-fertilization by sperm present in the initial inseminate. Re-insemination at 30 minutes resulted in re-fertilization of most of the eggs when dense sperm suspensions (1% and 0.5%) were used and less with the more dilute suspensions. The treatment with Ca- and Mg-free sea water resulted in generally higher percentages of re-fertilization but the effect in this set is slight.

In the second set of experiments listed in Table III the initial insemination was performed with more dilute sperm and the control polyspermy was lower (7%). Demembranation without reinsemination resulted in a small increase in percentage polyspermy (17% for the Ca- and Mg-free sea water-treated eggs and 13% for the untreated eggs). Upon re-insemination at 36 minutes the large increase in polyspermy was again obtained when relatively dense sperm suspensions were used and significant increases even in the dilute suspensions. Again the Ca- and Mg-free sea water-treated eggs give somewhat more re-fertilization than the untreated eggs. In this set inseminations of normal, unfertilized, eggs were made, at the same time and with the same sperm suspensions that were employed for the re-insemination. These controls (not listed in the table) gave 100 per cent fertilization in all but the 0.0063 per cent concentration of sperm (semen) which gave 90 to 95 per cent. Versene was not used in these experiments on *L. variegatus*.

Re-fertilization of trypsin-treated eggs

When unfertilized sea urchin eggs are treated with trypsin they fail to elevate a membrane upon fertilization as Runnström *et al.* (1943) originally showed. Hagström and Hagström (1954a) found that such eggs could be re-fertilized if

after initial insemination. All pictures taken at time of first cleavage of control eggs. Magnification 95 ×. (a) Control, non-demembranated eggs. (b) Control, demembranated eggs. Re-inseminated (c) at 10 minutes, (d) at 20 minutes, (e) at 40 minutes after first insemination. (f) Control eggs, singly inseminated in 0.03% semen, at 40 minutes after beginning of experiment.

TABLE III

Re-fertilization of mechanically demembranated eggs of Lytechinus variegatus with and without treatment with Ca- and Mg-free sea water; at 22° C.

Concentration of sperm (semen)		Percentage polyspermy	
First insemination, %	Second insemination at 30 minutes	Eggs exposed to Ca- and Mg-free sea water at 6 to 12½ minutes	Eggs washed in sea water at corresponding times
2	Not demembranated Not re-inseminated		10.5
2	Demembranated but not re-inseminated	45	58
2	1%	99.5	97
2	0.5	97.5	91
2	0.25	90	83.5
2	0.125	93	77.5
2	0.063	72.5	73.5
	Second insemination at 36 minutes	Ca- and Mg-free sea water at 5 to 11 minutes	
0.1	Not demembranated Not re-inseminated		7
0.1	Demembranated but not re-inseminated	17	13
0.1	0.1	74	86
0.1	0.05	77.5	56
0.1	0.025	60	52
0.1	0.0125	51	39
0.1	0.0063	44	40

TABLE IV

Re-fertilization of trypsin-treated eggs of Lytechinus pictus

First insemination; after 10 minute treatment of eggs with 0.01% trypsin in sea water and washing; Sperm per ml.	Second insemination		Percentage polyspermy
	Time of insemination in minutes	Sperm ($\times 10^6$) per ml.	
4×10^6	—	0	12
	5	8	47
	30	8	48
8×10^6	—	0	19
	5	8	73
	30	8	39
16×10^6	—	0	26
	5	8	76
	30	8	53

they were exposed to Ca- or Mg-free sea water for a short time at various times after the first fertilization. Practically no re-fertilization was obtained without the treatment with Ca- or Mg-free sea water.

We have found that when unfertilized eggs of *Lytechinus pictus* are exposed to trypsin, washed and inseminated, they can later be re-fertilized, without further treatment other than re-insemination. Table IV presents the results of a set of

TABLE V

Re-fertilization of papain-treated eggs of Lytechinus pictus

First insemination; eggs added to papain solution containing sperm; sperm per ml.	Time of papain treatment (minutes)	Second insemination (after washing eggs with sea water)		Percentage polyspermy
		Time in minutes	Sperm ($\times 10^6$) per ml.	
2×10^6	6	—	0	1
	6	14	2	2
	6	14	10	75
	6	40	2	2
	6	40	10	2
1.5×10^6	11	—	0	3
	11	18	1.5	10
	11	18	6	85
	11	40	1.5	8
	11	40	6	55
3×10^7	5	—	0	3
	5	7	30	95
	5	7	120	100
	10	—	0	5
	10	11	30	100
	10	11	120	100
	15	—	0	5
	15	17	30	98
	15	17	120	100
	20	—	0	18
	20	21	30	26
	20	21	120	100
	30	—	0	18
	30	32	30	100
	30	32	120	100
	40	—	0	17
	40	42	30	98
	40	42	120	100

experiments in which the eggs were treated for ten minutes with a solution of 0.01 per cent trypsin in sea water. After washing, three samples were inseminated, each with a different concentration of sperm (4×10^6, 8×10^6 and 16×10^6 per ml.). Samples from each of these were re-inseminated at 5 minutes and at 30 minutes. Trypsin-treatment is known (see Hagström and Hagström, 1954b; Tyler and Metz, 1955) to render sea urchin eggs susceptible to polyspermy. In the present experiment this is evident in the relatively high percentage (12, 19 and 26) of polyspermy

obtained without re-insemination. However, the data of the table show that upon re-insemination re-fertilization occurs as late as 30 minutes after initial fertilization. The re-insemination at 5 minutes is relatively more effective.

Re-fertilization of papain-treated eggs

Treatment of unfertilized sea urchin eggs with papain also destroys their ability to elevate a membrane upon fertilization. The fertilizability of the papain-treated eggs is, however, often rather poor. Tyler and Spiegel (1956) showed that fertilization proceeds very well if the eggs are added to a suspension of sperm in the papain solution. In these circumstances a rather unique phenomenon is observed; namely, a fertilization membrane is elevated, as fully as in normal sea water and then, within the next three or four minutes, retracts to the surface of the egg from which it is not distinguishable. Such eggs, even when soon removed from the papain solution, show no signs of a fertilization membrane at the time of cleavage.

In the present experiments *Lytechinus* eggs were added to sperm in papain solution. At various times after retraction of the fertilization membrane to the surface the eggs were washed in sea water and re-inseminated. The results of three sets of experiments are presented in Table V. The insemination in the papain solution results in a relatively low (1 to 5) percentage of polyspermy except in those experiments (in the third set) where the eggs were allowed to remain in the solution for 20 minutes or longer in which case it rose somewhat (17 to 18%). Upon re-insemination one hundred per cent polyspermy was obtained in many of the tests. As in the experiments with the mechanically demembranated eggs and the trypsin-treated eggs the re-fertilizability is higher the sooner the second insemination is done and the greater the concentration of sperm. This statement applies to comparisons within a set of experiments since in experiments performed on different days the absolute values may differ, as a comparison of the first two sets of experiments in Table IV indicates. If the eggs are allowed to remain in the papain solution until shortly before the re-insemination, as in the third set of experiments listed in Table IV, the re-fertilizability at the later times remains high. As in the mechanically demembranated eggs the degree of polyspermy following re-insemination is often too high to permit cleavage, the polyspermic nature of the eggs being indicated by the many asters present.

Discussion

It has been shown in the present experiments that fertilized eggs that have been removed from their fertilization membrane can be re-fertilized. This is contrary to the generally accepted views and the experience of many investigators (see Lillie, 1919; Just, 1939), the surface of the membrane-less fertilized egg being considered unreceptive to sperm. While the experiments of Tyler and Schultz (1932) demonstrated re-fertilizability of fertilized eggs this was confined to the period before membrane elevation occurs. The experiments of Sugiyama (1951) and of Hagström and Hagström (1954a, 1954b) showed that re-fertilization was possible at later stages, provided that the membrane-less eggs were exposed for a while to Ca- and/or Mg-free media. Without such treatment these workers obtained no significant re-fertilization of membrane-less eggs upon re-insemination. The dif-

ference in results may in part be due to differences in the species of echinoid used. In the present experiments the two preliminary experiments with *Dendraster* showed relatively little re-fertilizability in comparison with the results with *Lytechinus*. The difference may also be due in part to differences in the concentration of sperm used for re-insemination.

Sugiyama (1951) does not state the concentration of sperm used in his test (p. 336) of the re-fertilizability of denuded, untreated eggs of *Strongylocentrotus*. If it were 10^{-2} (one per cent semen; probably about 2×10^{-8} sperm per ml.), which, as shown in his Table I, was used for the eggs treated with Ca- and Mg-free sea water, this would be higher than concentrations found in the present experiments with *Lytechinus* to be capable of re-fertilizing practically all of the demembranated, untreated, eggs. Hagström and Hagström (1954a, 1954b) used 2×10^{6} sperm per ml. for re-inseminations of eggs of *Paracentrotus lividus* without getting significant re-fertilization of the controls. This is within the effective range for *Lytechinus* when the re-inseminations are performed early. It would appear, then, that species differences were largely responsible for the difference in results.

The use of Versene in the sperm suspension may have contributed to the effectiveness of the sperm in re-fertilization, by virtue of its favorable action (Tyler, 1953) on the fertilizing capacity of the sperm. However, it is not a determining factor in the present results, as the experiments (see Table II) in which it was omitted show. Also, it should be noted that in those experiments in which it was employed in the sperm suspension the amounts added with the sperm to the eggs were such as to make the concentration less than 10^{-5} molar.

One feature of *Lytechinus* eggs that might be related to the apparent ease with which they can be re-fertilized is the relative thinness, and slowness of formation, of the ectoplasmic (hyaline) layer. However, this layer is distinctly visible, and well formed, by about 15 minutes after fertilization, whereas re-fertilization is possible after 40 or more minutes.

It has been noted in the presentation of the data that the re-inseminated eggs tend to become highly polyspermic. This occurs with concentrations of sperm which, when used as a first inseminate, result in very little or no polyspermy. Evidently the demembranated fertilized egg does not possess the capability of the unfertilized egg of excluding all but one sperm. Experiments by Rothschild and Swann (1949, 1950, 1951, 1952; reviewed by Rothschild, 1954) have shown that the response of the unfertilized egg is characterized by a rapid change that passes over the surface in about two seconds, from the point of the entering sperm, that renders the surface about 95 per cent unreceptive to other sperm. This is followed by a change, requiring about 60 seconds, that makes the surface completely resistant. These changes are in advance of elevation of the fertilization membrane. However, the surface of the fertilization membrane is presumably this altered surface of the freshly fertilized egg. The surface of the denuded fertilized egg has evidently not become resistant to sperm and also lacks the ability to propagate a fast block to the entrance of more than one spermatozoon.

The ability of the denuded fertilized egg to be fertilized would imply that its surface possesses a sperm receptor substance similar to that of the unfertilized egg. In the latter this is attributed (see Tyler, 1948, 1955) to the substance known as fertilizin. It would be of interest to learn then whether the same kind of substance

is present on the surface of the denuded unfertilized egg. Experiments by Motomura (1950, 1953a, 1953b) have provided some evidence for the existence of a cytofertilizin within the egg. The evidence also points to the ectoplasmic (hyaline) layer of the fertilized egg as being the likely source. If the results are not due to possible incomplete removal of the fertilizin of the unfertilized egg, they provide then an interpretation of the re-fertilizability of the fertilized egg on the same basis as that of initial fertilization; namely that the specific adherence of the sperm is due to reaction of its anti-fertilizin with fertilizin of the egg. If the fertilizin-like substance is assumed to be present in lower concentration on the surface of the denuded fertilized egg than on that of the unfertilized egg, or if it is assumed to be less reactive, this would help explain why high concentrations of sperm are required for re-fertilization. Further, it would appear that this postulated fertilizin-like substance is only slowly lost or altered on the surface of the denuded fertilized egg, the egg being re-fertilizable for a considerable period. Since brief treatment with Ca- and/or Mg-free media permits re-fertilization at still later stages and such treatment partially dissolves the ectoplasmic (hyaline) layer it would further appear that the treatment exposes additional fertilizin-like material. Tests of these and related possibilities will be the subject of future investigations.

Summary

Fertilized, mechanically demembranated, eggs of the sea urchins *Lytechinus pictus* and *Lytechinus variegatus* can be re-fertilized when heavily re-inseminated at a half-hour or more (17° to 22° C.) after the initial fertilization. The re-fertilization occurs more readily the earlier the second insemination is performed. The re-inseminated eggs tend to become heavily polyspermic. Concentrations of sperm that give 100 per cent re-fertilization do not cause any significant polyspermy when used as a first inseminate on normal unfertilized eggs. The results show not only that the demembranated fertilized egg is capable of accepting additional sperm but also that it lacks the ability to propagate a fast block to all but one sperm. Treatment of the unfertilized eggs with trypsin, so as to suppress membrane formation, or with papain at the time of fertilization, so as to cause reversal of membrane elevation, also permits re-fertilization upon re-insemination without further treatment. The results are interpreted in terms of the possible presence of fertilizin-like sperm receptors on the surface of the denuded fertilized egg.

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